

## Color Tests

### 1 Introduction

Color tests are rapid toxicological qualitative methods used to establish the presence or absence of certain drugs based upon visible observation. The tests are primarily used to screen for the presence of drugs or metabolites in urine, but may also be used for other biological fluids (e.g., ~~urine~~, precipitated blood) and nonbiological samples (e.g., solids or liquids).

Positive results of a color test are only preliminary. A more specific analytical method must be used in order to obtain a confirmed analytical result. Gas chromatography/mass spectrometry (GC/MS) or liquid chromatography/mass spectrometry (LC/MS) are preferred confirmatory methods.

### 2 Scope

This procedure allows for screening of urine samples for acetaminophen, salicylic acid, trihalomethyl compounds (including chloral hydrate and its metabolites), and ethchlorvynol<sup>1</sup>. This procedure also allows for the screening of blood samples for acetaminophen and salicylic acid. Other drugs may be added to the routine screens as deemed necessary, provided the color tests are validated prior to use. This document applies to Chemistry Unit case working personnel who perform toxicology analyses.

### 3 Principle

Color tests involve the reaction of a specimen with a reagent or series of reagents to produce a change in color. The stronger the color change, the more drug or drug metabolite present in the sample.

- Acetaminophen and its metabolites are hydrolyzed in an acidic solution to p-aminophenol, which is coupled with o-cresol to give an indophenol blue color.
- Aspirin is hydrolyzed in an acidic solution to salicylic acid, which reacts with acidic ferric chloride solution to give a purple color.
- Trichlorinated compounds are polyhalogenated compounds. Compounds with at least two halogen atoms bound to the same carbon atom will react with pyridine to give a pink or deep yellow/orange color.
- Ethchlorvynol reacts with diphenylamine to form a purple color.

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<sup>1</sup> As of the date of this procedure, ethchlorvynol is not available on the legal drug market.

## 4 Specimens

This procedure has been validated for urine specimens. The acetaminophen and salicylate tests have been validated for blood specimens. Other matrices may be substituted provided appropriate controls are analyzed. A minimum of 1.7 mL of urine is needed to perform all four color tests. A minimum of 2.0 mL of blood is required to perform the salicylate and acetaminophen color tests.

## 5 Equipment/Materials/Reagents

- a. Deionized water
- b. Pyridine (certified A.C.S grade)
- c. Chloroform (HPLC grade)
- d. Hotplate – stirrer or Heating Block
- e. Pipettes (0.5 - 5 mL, 200-1000  $\mu$ L, 50-200  $\mu$ L) with disposable tips
- f. Test tubes (13 x 100 mm, 12 x 75 mm) with caps
- g. Vortexer
- h. 6 M Hydrochloric Acid (~ 50% v:v):  
To a 25-mL graduated cylinder containing 10 mL deionized water, add 12 mL concentrated hydrochloric acid and mix well. Bring to 24 mL with deionized water. Store in glass at room temperature. Stable 6 months.
- i. 2 M Ammonium Hydroxide:  
Add 10 mL concentrated ammonium hydroxide to 50 mL deionized water in a 100-mL graduated cylinder. Fill to the 75-mL mark with deionized water and mix well. Store in glass at room temperature. Stable 1 month.
- j. 1% (by volume) o-Cresol:  
Place 1 mL o-cresol in a 100-mL volumetric flask and fill to the mark with deionized water. Mix well and allow to stand for at least 24 hours before use. Store refrigerated in brown glass. Stable 6 months.
- k. Trinder's Reagent:  
Add 400 mg mercuric chloride, 400 mg ferric nitrate nonahydrate, and 0.1 mL concentrated hydrochloric acid to 5 mL deionized water in a 10-mL volumetric flask. Mix well to dissolve and bring to volume with deionized water. Store refrigerated in glass. Stable 3 months.

- l. 5 M (20% w/v) Sodium Hydroxide:  
To a 100-mL Nalgene volumetric flask, add 60 mL water and 20 g sodium hydroxide. Mix well to dissolve and bring to volume with deionized water. Store in Nalgene containers at room temperature. Stable 1 year.
- m. Balance
- n. Volumetric flasks
- o. Diphenylamine Reagent
- p. Methanol (HPLC Grade)
- q. Acetonitrile (HPLC Grade)
- r. 0.45  $\mu$ m Millipore Filter Tubes
- s. Centrifuge
- t. Evaporator with nitrogen

## 6 Standards and Controls

- a. Negative Control Urine:  
Obtained in house<sup>2</sup>. Store refrigerated in plastic.  
A Negative Control Urine will be analyzed every time a urine color test is performed.
- b. Negative Control Blood:  
Obtained in house or from a suitable vendor. Store refrigerated in glass or plastic.  
Stability determined by manufacturer.  
A Negative Control Blood will be analyzed every time a blood color test is performed.
- c. Acetaminophen:  
Purchased from U.S.P., or another approved vendor. Stability and storage determined by manufacturer.
- d. Acetaminophen Working Stock Solution (1 mg/mL):  
Place 10 mg acetaminophen in 10-mL volumetric flask, dilute to mark with methanol.  
Store refrigerated in glass, stable at least 6 months.
- e. Acetaminophen Positive Control Urine (100  $\mu$ g/mL):

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<sup>2</sup> Note: Synthetic urine (Surine) is unsuitable for the salicylate color test.

Add 1 mL of the Acetaminophen Working Stock Solution to a 10-mL volumetric flask, and bring to the mark with Negative Control Urine. Store refrigerated in glass or plastic. Stable at least 6 months.

The Acetaminophen Positive Control Urine will be analyzed with every urine acetaminophen color test.

- f. Acetaminophen Positive Control Blood (50 µg/mL):  
Add 0.5 mL of the Acetaminophen Working Stock Solution to a 10-mL volumetric flask, and bring to the mark with Negative Control Blood. Store refrigerated in glass or plastic. Stable at least 6 months.  
The Acetaminophen Positive Control Blood will be analyzed with every blood acetaminophen color test.
- g. Sodium Salicylate:  
Purchased from Sigma, or another approved vendor. Stability and storage determined by manufacturer.
- h. Sodium Salicylate Working Stock Solution (1 mg/mL):  
Place 10 mg sodium salicylate in 10-mL volumetric flask, dilute to mark with deionized water. Store refrigerated in glass. Stable at least 6 months.
- i. Sodium Salicylate Positive Control Urine (100 µg/mL):  
Add 1 mL of the Sodium Salicylate Working Stock Solution to a 10-mL volumetric flask, and bring to the mark with Negative Control Urine. Store refrigerated in glass or plastic. Stable at least 6 months.  
The Sodium Salicylate Positive Control Urine will be analyzed with every salicylate urine color test.
- j. Sodium Salicylate Positive Control Blood (50 µg/mL):  
Add 0.5 mL of the Sodium Salicylate Working Stock Solution to a 10-mL volumetric flask, and bring to the mark with Negative Control Blood. Store refrigerated in glass or plastic. Stable at least 6 months.  
The Sodium Salicylate Positive Control Blood will be analyzed with every salicylate blood color test.
- k. Trichloroacetic Acid:  
Purchased from Sigma, or another approved vendor. Stability and storage determined by manufacturer.
- l. Trichloroacetic Acid Working Stock Solution (1 mg/mL):  
Place 10 mg trichloroacetic acid in 10-mL volumetric flask, dilute to mark with methanol. Store refrigerated in glass, stable at least 6 months.
- m. Trichloroacetic Acid Positive Control Urine (50 µg/mL):  
Add 0.5 mL of the Trichloroacetic Acid Working Stock Solution to a 10-mL volumetric

flask, and bring to the mark with Negative Control Urine. Store refrigerated in glass or plastic. Stable at least 6 months.

The Trichloroacetic Acid Positive Control will be analyzed with every Fujiwara Color Test (Trichlorinated Compound Color Test).

- n. Ethylchlorvynol:  
Purchased from U.S.P., or another approved vendor. Stability and storage determined by manufacturer.
- o. Ethylchlorvynol Working Stock Solution (100 µg/mL):  
Place 100 uL ethylchlorvynol in a 12 x 75 mm test tube, add 900 uL of methanol. Store refrigerated in glass, stable at least 6 months.
- p. Ethylchlorvynol Positive Control Urine (30 µg/mL):  
Add 1 mL of the Ethylchlorvynol Working Stock Solution to a 10-mL volumetric flask, and bring to the mark with Negative Control Urine. Store refrigerated in glass or plastic. Stable at least 6 months.  
The Ethylchlorvynol Positive Control will be analyzed with every Ethylchlorvynol Color Test.

## 7 Sampling

Not applicable.

## 8 Procedure

Appendix 1 contains an abbreviated version of this procedure. This form may be used at the bench by the Examiner or Chemist performing the procedure.

Results of the following tests should be photographed for documentation and review.

- a. Acetaminophen Color Test:
  - 1. Preparation of blood samples:
    - a. Add 1 mL of blood (sample or control) to a 13 x 100 test tube.
    - b. While vortexing, add 2 mL acetonitrile, dropwise.
    - c. Transfer supernatant to a 0.45 µm Millipore filter tube.
    - d. Centrifuge at approximately 4000 rpm for 15 minutes.

- e. Transfer filtrate to a new test tube, and evaporate to dryness under nitrogen at 35°C.
  - f. Reconstitute residue in 0.5 mL deionized water. Vortex well.
2. Place 0.5 mL of urine or reconstituted blood extract (sample or control) in a properly labeled test tube (13 x 100 mm). [Note: test also works at 1/5 volumes in a 10 x 75 mm test tube.]
  3. Add 0.5 mL of 6 M Hydrochloric Acid Solution to sample.
  4. Vortex samples for 10 - 15 seconds, and then place them in a boiling hot water bath or heating block at 98°C. Remove from water bath/block after 10 minutes.
  5. Add 3 mL of deionized water.
  6. Add 2 mL of 2 M Ammonium Hydroxide Solution.
  7. Add 0.5 mL of O-cresol Solution (1%).
  8. Wait 10 - 25 minutes. (Wait a minimum of 25 minutes if acetaminophen and salicylate are expected in a blood extract.)
  9. View color. A blue color (indicative of acetaminophen) will appear with a positive result.
- b. Salicylate Color Test - (Trinder's color test):
1. Preparation of blood samples:
    - a. Add 1 mL of blood (sample or control) to a 13 x 100 test tube.
    - b. While vortexing, add 2 mL acetonitrile, dropwise.
    - c. Transfer supernatant to a 0.45 mm Millipore filter tube.
    - d. Centrifuge at approximately 4000 rpm for 15 minutes.
    - e. Transfer filtrate to a new test tube, and evaporate to dryness under nitrogen at 35°C.
    - f. Reconstitute residue in 0.2 mL deionized water. Vortex well.
  2. Place 0.2 mL of urine or reconstituted blood extract (sample or control) in a

properly labeled test tube (12 x 75 mm).

3. Add 0.2 mL of Trinder's Reagent.
4. Vortex for 10 - 15 seconds.
5. Wait 5 minutes.
6. View color. A tan/brown (indicative of methylsalicylate) or tan/purple (indicative of sodium salicylate) will appear with a positive result.

c. Trichlorinated Compound Color Test - (Fujiwara color test):

1. Place 0.5 mL of urine (sample or control) in a properly labeled test tube (12 x 75 mm).
2. Add 0.5 mL of 20% Sodium Hydroxide Solution.
3. Add 0.5 mL of pyridine.
4. Vortex samples for 10 - 15 seconds, and then place them in a boiling hot water bath or heating block at 98°C. Remove from water bath/block after 10 minutes.
5. View color in the top (pyridine) layer. A pink (indicative of chloral hydrate or trichloroacetic acid) or deep yellow/orange (indicative of 2,2,2-trichloroethanol) will appear with a positive result.

d. Ethchlorvynol Color Test:

1. Place 0.5 mL of urine (sample or control) in a properly labeled test tube (12 x 75 mm).
2. Add 0.25 mL of diphenylamine reagent.
3. Vortex for 10 - 15 seconds.
4. Wait 5 minutes.
5. Add 1 mL of chloroform.
6. Vortex samples for 10 - 15 seconds.
7. Wait approximately 1 minute.

8. View color. A purple color (indicative of ethchlorvynol) will appear in the lower (chloroform) layer with a positive result, or blue (indicative of negative specimen) will appear in the lower (chloroform) layer with a negative result.

## 9 Decision Criteria

Generally speaking, a specimen is considered to give a positive response for a particular drug class if the specimen compares favorably to the corresponding positive control.

- a. Acetaminophen Color Test: Blue color indicates positive result for acetaminophen.
- b. Salicylate Color Test: Tan/brown color indicates a positive result for methylsalicylate, tan/purple color indicates a positive result for sodium salicylate.
- c. Trichlorinated compounds Color Test: Pink (or very deep red-purple for strong positive) color in upper (pyridine) layer within minutes indicates a positive result for chloral hydrate or trichloroacetic acid. Deep yellow/orange color in top layer indicates a positive result for 2,2,2-trichloroethanol. Note: May not be a good indicator for 2,2,2-trichloroethanol since positive result (deep yellow/orange color) may be hard to differentiate from the color of urine specimen, even with analysis of a negative control.
- d. Ethchlorvynol Color Test: Purple color in the lower layer within 2-3 minutes indicates a positive result for ethchlorvynol.

It is the ultimate judgment of the Examiner to determine if the results suggest the need for additional testing. If a specimen is deemed positive for a particular drug class, a separate analysis utilizing a different principle must be performed in order to confirm the presumptive positive.

## 10 Calculations

Not applicable.

## 11 Measurement Uncertainty

Not applicable.



## 12 Limitations

### a. Limits of Detection (LOD):

1. Acetaminophen Color Test: 40 µg/mL for acetaminophen in urine  
10 µg/mL for acetaminophen in blood
2. Salicylate Color Test: 300 µg/mL for methylsalicylate in urine  
45 µg/mL for sodium salicylate in urine  
10 µg/mL for sodium salicylate in blood
3. Trichlorinated Compounds Color Test: 10 µg/mL chloral hydrate  
4 µg/mL trichloroacetic acid
4. Ethchlorvynol Color Test: 25 µg/mL ethchlorvynol

### b. Sensitivity:

1. Acetaminophen, Salicylate, and Ethchlorvynol Color Tests:  
Positive results can be obtained with therapeutic doses.
2. Trichlorinated Compounds Color Test:  
Positive results can be obtained with therapeutic doses in plasma and with overdoses in urine.

### c. Specificity:

1. Acetaminophen Color Test:  
Positive results may be obtained with phenacetin (acetaminophen and p-aminophenol are metabolites of phenacetin, which is not available within the US). False negative results may occur in patients with ketonuria. When both acetaminophen and salicylate are present in a blood sample, false negative results may be obtained if the wait time is not lengthened to 25 minutes.
2. Salicylate Color Test:  
False positive results may be obtained from the following compounds: aminosalicilic acid, diflunisal, labetalol. When both acetaminophen and salicylate are present in a blood sample, false negative results may be obtained for low levels of salicylate (less than 50 PPM).
3. Trichlorinated Compounds Color Test:  
Positive results indicate the presence of a polyhalogenated compounds. Polyhalogenated compounds that may also give positive results include: bromoform, carbon tetrachloride, chloramphenicol, chlorbutol, chlorobutanol,

chloroform, DDT, ethyl bromide, ethylene chloride, iodoform, methyl bromide, methylene chloride, pentachloroethylene, perchloroethylene, tetrachloroethane, tribromoethanol, and trichloroethylene.

4. Ethchlorvynol Color Test:  
False positive results may be obtained from phenothiazines.

### 13 Safety

Take standard precautions for the handling of chemicals and biological materials. Refer to the *FBI Laboratory Safety Manual* for proper handling and disposal of all chemicals.

### 14 References

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*FBI Laboratory Safety Manual.*

Rev. #	Issue Date	History
0	01/30/06	New document.
1	09/08/08	Updated title. Added note that ethchlorvynol no longer available on US drug market in section 2. Expanded sections 2, 4, 5, 6, 9 and 14 to cover newly validated analysis of blood samples for acetaminophen and salicylate testing. Clarified in-house urine in section 6a. Updated preparation of Trichloroacetic Acid Positive Control Urine in section 6. Extended wait time for Acetaminophen Color Test to 25 minutes.
2	02/09/18	Updated Scope language. Updated Sections 9a.4 and 9c.4 to allow for use of heating block. Updated Section 5d and Appendix 1 to include heating block. Updated approval lines. Removed footer. Removed Calibration section (formerly 7), and updated numbering. Changed to 'Measurement Uncertainty' for Section 12. Section 6a: removed expiration for urine control. Removed references to TOX103 in Section 5 and References, and updated 5: h.-l. Deleted Instrumental Conditions (formerly Section 9) and renumbered.

**Approval**

Redacted - Signatures on File

Toxicology  
Technical Lead:

Date: 02/08/2018

Chemistry Unit Chief:

Date: 02/08/2018

**QA Approval**

Quality Manager:

Date: 02/08/2018

**Appendix 1: Abbreviated version of the Color Test Procedure for bench use.**

Redacted - Form on File